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14. ABSTRACT
We developed electrophysiological and pharmacological studies to investigate the effects of levetiracetam, topiramate and carbamazepine in excitatory (glutamatergic) synaptic transmission onto granule cells in the dentate gyrus from slices of control and pilocarpine-treated epileptic rats and mice. We discover a novel presynaptic action of topiramate reducing the frequency of mEPSC in a dose-dependent manner while the inhibitory action on mEPSC amplitude was also present in both control and epileptic slices. Hence, our findings indicate that topiramate exert, at least in part a presynaptic action inhibiting the release of glutamate.We detected that levetiracetam inhibits the spontaneous glutamate release (mEPSC frequency) in control and epileptic rats and mice. In addition, levetiracetam was more effective in reducing excitatory synaptic transmission onto dentate granule cells in slices from chronically epileptic rats while no effect was detected on the amplitude of mEPSC indicating no action of post-synaptic AMPA receptors. We also detected that LEV increase the GABAergic inhbnitory transmission onto dentate granule cells by increasing the frequency of mIPSCs. These data indicate that presynaptically acting drugs as levetiracetam may become a key piece in the arsenal of antiepileptic drugs in mesial temporal lobe epilepsy. Thereby, screening for a presynaptic action site may be part of the strategy to discover novel and effective antiepileptic drugs.

15. SUBJECT TERMS
Epilepsy, Presynaptic, antiepileptic drugs, levetiracetam, topiramate, carbamazepine, synaptic vesicle proteins, seizures.

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SECOND YEAR PROGRESS REPORT: DoD Grant - W81XWH-11-1-0356

Title: "*New Treatments for Drug-Resistant Epilepsy that Target Presynaptic Transmitter Release*".

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INTRODUCTION: Post-traumatic epilepsy (PTE) is a major long-term complication of traumatic brain injuries (TBIs), which are often suffered by members of the Armed Forces. PTE usually develops within five years of a head injury, and it is often expressed as medically intractable hippocampal epilepsy. Although there are a variety of causes of traumatic epilepsy, the resulting chronic neurological condition is characterized by common features, including recurrent spontaneous seizures, neuronal damage, mesial temporal lobe epilepsy (MTLE) in ~30% of patients, and resistance to available anticonvulsant drugs. Therefore, it is of critical importance to develop novel models to study post-traumatic epilepsy in order to facilitate the discovery of new treatments. **Background:** during epileptogenesis, seizure-related functional and structural reorganization of neuronal circuits leads to both hyperexcitability of glutamatergic neurons and defective inhibition. While many postsynaptic alterations have been demonstrated, there is surprisingly little known concerning dysfunction of presynaptic transmitter release machinery in epilepsy. The recent successful introduction of the antiepileptic drug levetiracetam (LEV), which acts on presynaptic molecular targets, suggests that controlling dysregulation of presynaptic function could be a promising new therapeutic target for the treatment of unresponsive epilepsies. While LEV has been shown to bind to both the synaptic vesicle protein SV2a and N-type Ca^{2+} channels, its precise mechanism of action is not understood. Recent studies have found that severe seizures can down-regulate the expression of both SV2a and the group II metabotropic glutamate (mGluRII) autoreceptor that normally control glutamate release from presynaptic terminals.

HYPOTHESIS AND OBJECTIVES: During periods of intense neuronal activity such as seizures, a larger pool of vesicles could result in more glutamate being released and long-lasting aberrant excitation. We propose to explore the effects of seizures on transmitter release and the presynaptic action of AEDs on these changes. We will use electrophysiology and multiphoton confocal microscopy. Preliminary data indicate that SE induces long-lasting potentiation of synaptic vesicle release in epileptic rats. We hypothesize that successful AED treatment might prevent or reverse these seizure-induced molecular deficiencies (reduction of N-type VGCC, mGluR II and SV2a expression), and be antiepileptogenic as well. Our **central hypothesis** is that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to ameliorate epileptogenesis and excessive synaptic excitation in epilepsy.

The **long-term objectives** of this collaborative proposal are to: (1) investigate whether down-regulation of SV2a is responsible for reducing the anticonvulsant efficacy of LEV (this phenomenon is known as tolerance and has limited the use of LEV in the treatment of epilepsy), (2) identify the most effective AEDs which modulate presynaptic glutamate release, and (3) determine the presynaptic mechanism of action of the new AED LEV to modulate vesicular release properties. *We predict that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to treat many cases of drug-resistant epilepsy, especially epileptogenesis following traumatic brain injury.* In Year 2 of this proposal, we developed experiments to accomplish the specific 2 (below) and support imaging data on release of glutamate.

Specific Aim 2: *Assess whether antiepileptic drugs acting on presynaptic sites can reduce or prevent seizure induced long-term potentiation of vesicular release from mossy fiber boutons in MTLE.* **Working hypothesis:** Epileptic rats exhibit enhanced pool size and release probability from the rapidly-recycling vesicle pool, and SV2a down-regulation contributes to this enhanced release. Chronic treatment with LEV or other presynaptic antiepileptic drugs during epileptogenesis will protect presynaptic function and normal glutamate release, reducing or preventing hyperexcitability and seizures (months 13-24).

BODY: Description of Research Accomplishments toward accomplishing the aims.

1. Infrastructure upgrade at Dr. Garrido's site

*New Laser Scanning Confocal Microscope for functional imaging and physiology***:** During the period of the second year of the grant, infrastructure for research at Dr. Garrido's site was further enhanced with a significant upgrade of the Imaging Core facilities. The imaging core acquired a new imaging and physiology workstation with a spectrum-based laser scanning confocal microscope TSC SP2 (Leica) to add to the resources available for this project. This core facility is conveniently located in front of Dr. Garrido's laboratory. This microscope is suitable for functional imaging including analysis of stimulus-evoked changes in fluorescence of SynaptopHlourin (SpH) in transgenic mice and it will positively contribute to the overall performance of this project since confocal capabilities are now excellent at both sites.

2. Development of the pilocarpine model of epilepsy in mice and rats

Model of epilepsy in SPH mice: During the second year, we continued developing and improving the pilocarpine model of epilepsy in mice and rats at both institutions. During year 1, we acquired SpH transgenic mice from Jackson Laboratories to establish a newly refreshed colony and and develop the pilocarpine model of epilepsy in parallel with Dr. Stanton's laboratories. For our surprise, animals obtained from Jackson laboratories, Inc exhibited an increased resistance to enter status epilepticus in contrast to colonies established at Dr. Stanton's laboratories based in animals originally provided by Dr. Venkatesh N. Murphy (Harvard University). These negative results contrasted with our collaborative studies performed at Dr. Stanton's lab using SpH mice. Thereby, we further investigated the source of this variability. For developing the SpH pilocarpine model of epilepsy at Dr. Garrido's site, breeders were obtained from Jackson Laboratories *i.e.* B6.CBA-Tg(Thy1-spH)21Vnmu/J (Stock Number: 014651). The difference of these mice with Dr. Stanton SpH mice was that at Jackson Laboratories original SPH mice (donated by Dr. Murphy) were cross-bred with C57BL/6J (Stock No.o 000664). In recent studies, C57BL/6J mice have been found to be highly resistance to pilocarpine-induced seizures and status epilepticus[1]. To address this problem, Dr. Stanton sent original SPH mice in his colony to Dr. Garrido's site. Breeding of those mice has been successful and we are now expanding the colonies to increase the number of animals to continue the imaging studies, specially using the new imaging capabilities.

Model of chronic epilepsy in rats: All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with the approval of The University of Texas at Brownsville Institutional Animal Care and Use Committee (Protocol #2004-007-IACUC-1). Male Sprague–Dawley rats were maintained in a temperature- and humidity-controlled vivarium, with water and standard laboratory chow ad libitum. All efforts were made to minimize the number of animals in the study. A subset of the animals was made chronically epileptic by the systemic injection of pilocarpine, as described elsewhere[2-6].

3. Effect of LEV in network excitability and excitatory transmission in control and chronically epileptic rats.

We investigated whether acute or chronic treatment of slices with LEV will affect the excitability in hippocampal slices obtained from control and chronically epileptic rats. For this purpose, we performed extracellular recordings of population spikes in CA1 area of hippocampus and field excitatory postsynaptic potentials in dentate gyrus. In previous year, we demonstrated that LEV significantly reduced the frequency of excitatory postsynaptic currents onto granule cells of dentate gyrus in both mice and rats.

Brief description of methods for hippocampal slice preparation: Brains from >60 days of status epileptic or control Sprague Dawley rats were used for all experiments, and procedures were approved by The University of Texas at Brownsville Institutional Animal Care and Use Committee (Protocol 2011-001-IACU). After isoflurane anesthesia, rats were decapitated and their brains were quickly removed and submerged in 0 C artificial cerebral spinal fluid (ACSF) solution containing (in mM) 124 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 25 $NaHCO_3$ and 10 glucose. For preparation the hippocampal slices, the whole brain excluding the olfactory bulbs was rapidly removed after decapitation and immediately in cooled in oxygenated ice-cold ACSF. Horizontal hippocampal slices were cut at 350 μm using Leica Vibratome. The experiments were performed in slices from control and epileptic rats. To assess the effect of LEV in excitability and the antiepileptic action, slices were pre-incubated for 3 hours in ACSF solution containing 50μM AP5 to block NMDA receptors. A group of slices (Non-treatment) was incubated with this baseline sACSF solution while another group of slices from same animal was incubated with a treatment ACSF solution containing 300 μM LEV (Sigma Aldrich). After 3 h incubations slices were transferred to recording chamber (32^0C) and perfused at 2ml/min with oxygenated ACSF, or 300μM LEV (treatment).

3.1. Effects of LEV on population spikes evoked in CA1 area of hippocampal slices.

In these experiments LEV treatment resulted in a reduction of the amplitude of CA1 population spikes in both control and epileptics but this effect was not significant at least for our sample size. The effect of LEV in CA1 excitability of epileptic group was more pronounced (28.5% reduction, Fig. 1b2, Table 1.2) in contrast to effect on controls (20.7% reduction, Figure 1a2, Table 1.1). Population spikes in CA1 evoked for stimulation of the Shaffer collaterals were recoded and averaged in the Fig 1A and Fig 1B for the control and epileptic group respectively. The test stimulus intensity was adjusted to evoked 40% the maximal response of the input output (I/O). Levetiracetam did not have any significant effect on the mean population spike amplitude, slope and coastline in the control group (n=10) or in the chronic epileptic group (n=3). Synaptic responses in slices from the control and epileptic group were clearly altered after treatment with LEV (Figure 1A-a2 and 1B-b2) respectively. Although the inhibitory effect of LEV was very pronounced, analysis failed to reveal a statistical significance in neither of both groups. Paired t test analysis revealed, no significant effect were found in control group (n=10) in the amplitude (t=-09396, df=9, p= 0.37195), Coastline (t= 1.35339, df =9 p= 0.20894) and Slope (t=-100405, df=9 , p= 0.34159) before or after the application the 300μM the LEV. In addition, no significant effect in the effect of LEV treatment in slices from the epileptic group

(SE-LEV) (n=3) was found in the analysis of the amplitude (t=-0.856, df=2, p=0.48868), coastline (t=0.24009, df=2, p=0.83262) or slope (t=-1.84803, df=3, p=0.20585) when compared with effect of LEV in slices of control group. These data indicate that potential antiepileptic action of LEV in chronically epileptic tissue is preserved despite seizure-mediated down-regulation of SV2A proteins as measured by immunohistochemistry, Western blotting and real-time quantitative PCR (see below). Understanding the antiepileptic effect of LEV despite down-regulation of pharmacological targets is important to design similarly effective drugs acting on presynaptic sites.

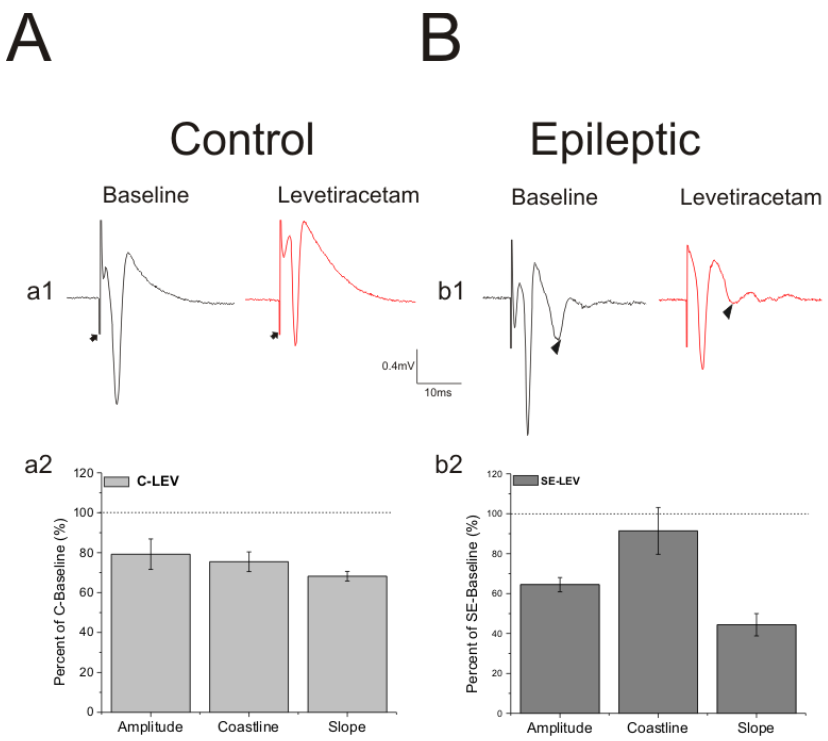

Figure 1. *LEV reduced amplitude of population spike responses evoked in the CA1 area of hippocampal slices.* (1A-1B). Representative recordings of population spikes show a reduction in amplitude after LEV treatment in control group (a1) control-baseline (black trace) compared to control-LEV (red trace) and (b1) epileptic(SE)-group where baseline (black trace) is compared to LEV treatment (SE-LEV, red trace). Graphs in a2 and b2 summarize percent changes of population spike amplitude, coastline and slope after LEV treatment in control and epileptic group respectively. Note reduction in the second population spike in chronic epileptic rat is observed (black triangle). Arrow shows stimuli artifacts.

Table 1.1 Paired samples *t*-test analysis for population spikes in CA1 area in control group

Population spikes Control (CA1)

		N	Mean	SD	SEM	t Stat	DF	Prob>|t|	% change
Amplitude	**C-Baseline**	10	-0.9877	0.48505	0.15339	-0.9396	9	0.37195	20.70%
	C-LEV	10	-0.7823	0.36989	0.11697				
	Difference		-0.2054						

		N	Mean	SD	SEM	t Stat	DF	Prob>|t|	% change
Coastline	**C-Baseline**	10	4.4394	2.17127	0.68662	1.35339	9	0.20894	24.55%
	C-LEV	10	3.3495	1.07123	0.33875				
	Difference		1.0899						

		N	Mean	SD	SEM	t Stat	DF	Prob>|t|	% change
Slope	**C-Baseline**	10	-1.4337	1.38163	0.43691	-1.00405	9	0.34159	31.17%
	C-LEV	10	-0.9779	0.33317	0.10536				
	Difference		-0.4558						

Table 1.2 Paired t-test analysis for population spikes in CA1 area in epileptic group

Population spikes Epileptic Group (CA1)

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|	% change
Amplitude	**SE-Baseline**	3	-0.856	0.62521	0.36097	-0.84143	2	0.48868	28.5
	SE-LEV	3	-0.612	0.19948	0.11517				
	Difference		-0.244						

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|	% change
Coastline	**SE-Baseline**	3	3.697	1.97978	1.14302	0.24009	2	0.83262	7.3
	SE-LEV	3	3.426	1.29803	0.74942				
	Difference		0.271						

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|	% change
Slope	**SE-Baseline**	3	-1.083	0.97686	0.56399	-1.84803	2	0.20585	39.5
	SE-LEV	3	-0.558	0.49915	0.28818				
	Difference		-0.525						

3.2. Effect of LEV on excitatory transmission in dentate gyrus in hippocampal slices from control and epileptic rats.

In this part of the study, we developed electrophysiology experiments to correlate data and understand mechanisms of inhibitory effect of LEV in presynaptic as detected by two-photon imaging of presynaptic release in SpH mice and patch-clamp analysis of excitatory postsynaptic currents. Our previous study indicates a long-term enhancement of presynaptic glutamatergic transmission in chronically epileptic mice and rats[2]. In this project, we are investigating if LEV can restore normal levels of presynaptic transmission at excitatory synapses by reducing release of glutamate. Accordingly, in order to assess the frequency-dependent action of LEV on presynaptic transmission, we implemented a paradigm consisting on recording field excitatory postsynaptic potentials (fEPSPs) during repetitive stimulation at a frequency of 20 Hz (10 stimuli) of perforant path afferents to granule cells in dentate gyrus of hippocampal slices maintained in vitro.

LEV significantly reduced the amplitude of fEPSPs in dentate gyrus of slices from both control (Fig. 2A) and epileptic groups (Fig. 2B) in response to repetitive stimulation (10 stimuli train at 20 Hz). The fEPSP responses were averaged for 10 min in two conditions: (a) 3h pre-incubation and perfusion with ACSF containing 50 μM AP5 (black traces) and (b) 300 μM LEV solution containing 50M AP5 (red traces) (Fig. 2 a1,b1). As previously reported for dentate gyrus[7,8], stimulation of the perforant path induced a paired pulse depression of subsequent fEPSP responses at 50 ms intervals, a phenomenon that was more evident during the first 4 fEPSPs in the train (Fig. 2 a1). Pre-incubation (3h) and perfusion with LEV (300 μM) induced a significant reduction of fEPSP amplitudes in the train in control slices (Fig. 2 a2). The summated data of all fEPSPs in the train revealed a significant 37.6 % reduction of mean summated fEPSP amplitudes after LEV treatment compared to slices (n=6) exposed to only ACSF and 50 μM AP5 (Graph inset in Fig. 2 a2, Table 2.1). The statistical analysis comparing relative changed in baseline versus LEV treatment for each fEPSP in the train revealed that LEV significantly reduced the amplitude for all individual fEPSPs in the train (Table 2.2). In contrast to control fEPSP responses, 2 slices out 6 from epileptic group exhibited paired pulse facilitation of second fEPSP ($fEPSP_2$) (see Fig. 2 b1) while 4 slices exhibited paired-pulse depression (Fig. 3). However, the analysis of the mean fEPSP amplitude revealed an overall depression of second fEPSP in the epileptic group (fig. 2 b2, black squares). Similarly to controls, LEV treatment resulted in a significant decrease in the amplitude of the fEPSP in treated slices when compared untreated slices (Fig. 2 b1, b2). This inhibitory action of LEV on fEPSP amplitude was detected in (a) the mean summated amplitude of fEPSPs in the train (a significant 49% reduction, paired student t-test, $p<0.0001$, Fig. 2. B2 inset graph, Table 2.1) and (b) for each individual fEPSPs in the train (Table 2.2). Statistical analysis revealed that LEV induced a significant 33.6% more reduction of fEPSP's amplitudes in slices from epileptic rats (Student t-test, $p<0.01$) when compared to the effect of LEV in fEPSPs in control group.

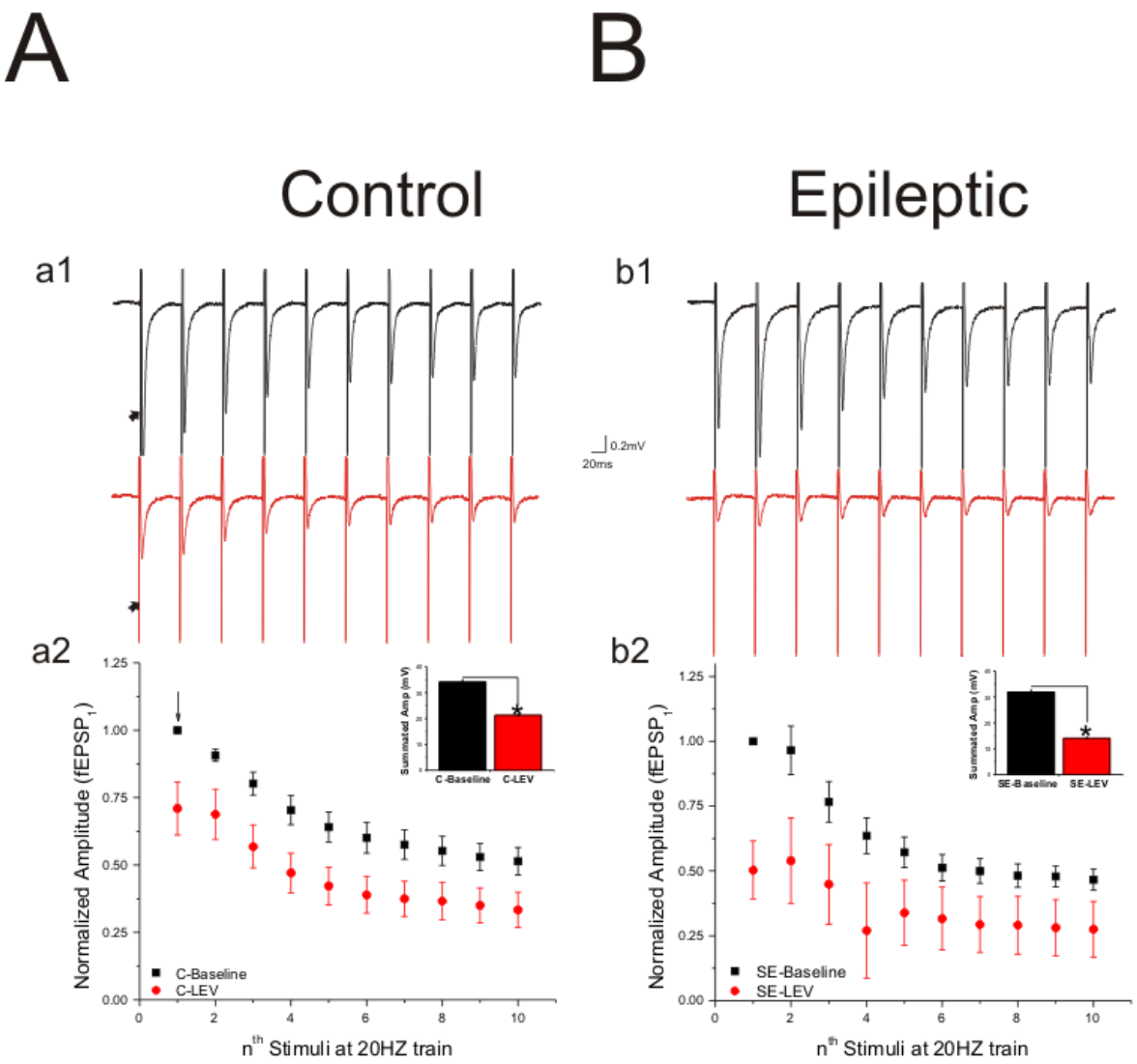

Figure 2. *LEV reduce excitatory transmission of perforant path to dentate gyrus in both control (A) and epileptic groups (B)*. Upper raw illustrate representative traces of dentate gyrus fEPSP responses to 20Hz stimulation (10 stimuli) in untreated (black) and 300 μM LEV treated (red) slices from control (a1) versus epileptic (b2) rat. Notice a marked reduction in fEPSP amplitudes during the train after LEV treatment. Graph representation of normalized mean fEPSP amplitude in untreated and LEV treated slices (Red symbols) show a significant reduction of amplitude of fEPSP in the train after LEV treatment in slices obtained from both control (Aa2) and epileptic group (Bb2).

Table 2.1 Paired samples *t*-test analysis for summered amplitudes of dentate gyrus repetitive stimulation on control and chronic epileptic rat

	N	Mean	SD	SEM	t Statistic	DF	Prob>|t|	% change
C-Baseline	10	-0.5684	0.15658	0.04951	-14.0109	9	0.0000002	**37.6**
C-LEV	10	-0.35439	0.11041	0.03492				
Difference		-0.21401						

	N	Mean	SD	SEM	t Statistic	DF	Prob>|t|	% change
SE-Baseline	10	-0.53226	0.13954	0.04413	-9.25131	9	0.0000068	**49**
SE-LEV	10	-0.27143	0.06196	0.01959				
Difference		-0.26084						

Table 2.2 Balance Two-Way Repeated Measures ANOVA to analyze the effect of LEV on individual fEPSPs in train evoked by 20Hz stimulation in control and epileptic groups.

Control Group	peaks	Mean Difference	Std. Error	DF	|t|value	Prob>|t|	Alpha	Sig Flag	% change
Baseline LEV	2	0.21904	0.04856	40	6.37969	0.0000554	0.05	1	**24.15**
Baseline LEV	3	0.23372	0.04856	40	6.80721	0.0000214	0.05	1	**29.15**
Baseline LEV	4	0.23269	0.04856	40	6.77716	0.0000229	0.05	1	**33.09**
Baseline LEV	5	0.2187	0.04856	40	6.36978	0.0000566	0.05	1	**34.13**
Baseline LEV	6	0.21206	0.04856	40	6.17644	0.0000866	0.05	1	**35.28**
Baseline LEV	7	0.2011	0.04856	40	5.85709	0.0001732	0.05	1	**34.94**
Baseline LEV	8	0.18643	0.04856	40	5.42987	0.0004299	0.05	1	**33.73**
Baseline LEV	9	0.17949	0.04856	40	5.22763	0.0006553	0.05	1	**33.88**
Baseline LEV	10	0.18088	0.04856	40	5.2684	0.0006022	0.05	1	**35.17**

Epileptic- Group	peaks	Mean Difference	Std. Error	DF	|t|value	Prob>|t|	Alpha	Sig Flag	% change
Baseline LEV	2	0.42612	0.06684	48	9.01562	0.0000002	0.05	1	**44.14**
Baseline LEV	3	0.31758	0.06684	48	6.71918	0.0000188	0.05	1	**41.44**
Baseline LEV	4	0.26813	0.06684	48	5.67294	0.0002104	0.05	1	**42.19**
Baseline LEV	5	0.23293	0.06684	48	4.92829	0.0010600	0.05	1	**40.70**
Baseline LEV	6	0.19558	0.06684	48	4.13801	0.0052300	0.05	1	**38.19**
Baseline LEV	7	0.20635	0.06684	48	4.36578	0.0033500	0.05	1	**41.29**
Baseline LEV	8	0.19119	0.06684	48	4.04502	0.0062500	0.05	1	**39.67**
Baseline LEV	9	0.19724	0.06684	48	4.17318	0.0048900	0.05	1	**41.22**
Baseline LEV	10	0.19063	0.06684	48	4.03336	0.0063900	0.05	1	**40.89**

In order to assess the effect of LEV in frequency-dependent changes in short-term synaptic plasticity of excitatory transmission we tested if LEV treatment (300 μM) on paired-pulse ratios (PPR) during the duration of the train in control versus epileptic group. Although LEV treatment reduce the overall fEPSP amplitude (Fig. 3A a2,b2), when PPR2 was analyzed ($fEPSP_2/fEPSP_1$), LEV treatment (n=6) reduced the PPR_2 (less depression) relative to the first $fEPSP_1$ when compared to baseline (no treatment, n=6) in control group (Fig. 3A a1) but this effect was not statistically significant (Fig. 3A a2) (Paired t-test, P>0.05). In the epileptic group, the effect of LEV (n=6) on PPR_2 was more complex since LEV induced less depression of PPR_2 in 5 out 6 slices, but in 1 slice, LEV induced more depression (Fig. 3B b1) when compared to basleine no treatment (n=6). Overall, there were not significant changes in PPR_2 in this group (Fig. 3B b2) (Paired t-test, P>0.05).

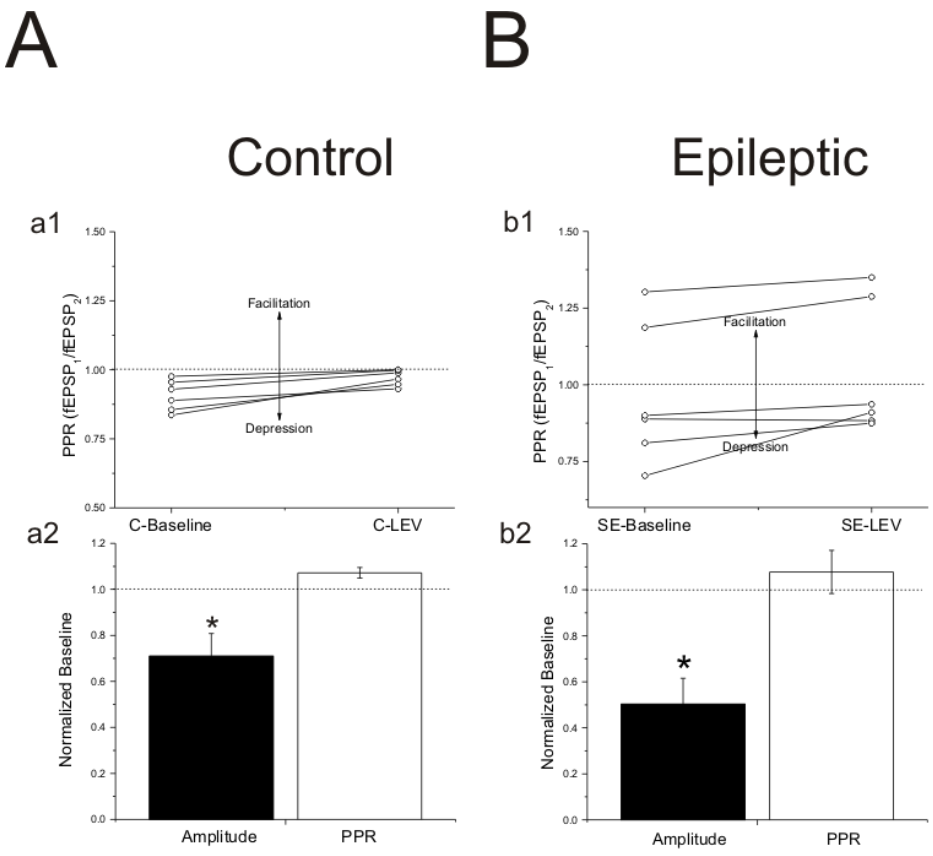

Figure 3. *Effect of LEV on paired-pulse ratio of perforant parth to dentate gyrus excitatory synapses in control (A) versus epileptic groups (B)*. Aa1. Graph representing a paired-pulse depression of PPR for first two responses (fEPSP2/fEPSP1) (in slices non treated (C-Baseline) and a reduction of depression (relative facilitation) after treatment with 300 μM LEV (C-LEV). Bb1. Graph represented a complex behavior of PPR in untreated slices (4 depression and 2 facilitation). Similarly to control slices, treatment with LEV (SE-LEV) a reduced depression (relative facilitation) was observed in all the slices. Aa2. Summary graph representing a reduction of amplitude of first fEPSP and a non-significant increase in relative facilitation in control group after treatment with LEV. Bb2. Summary graph representing a significant reduction of amplitude of first fEPSP and a non-significant increase in relative facilitation in epileptic group following treatment with LEV.

The individual analysis of depression of fEPSP2 in all slices of control group revealed that all except one slice exhibited paired-pulse depression with or without LEV treatment (Fig. 4A a1, a2). In contrast to control group, 4 out 12 slices exhibited paired-pulse facilitation *i.e.* 2 slices in baseline SE non-treated group, Fig. 4B b1) and 2 slices in LEV treated epileptic group (Fig. 4B b2) and 8 slices exhibited depression in the epileptic group.

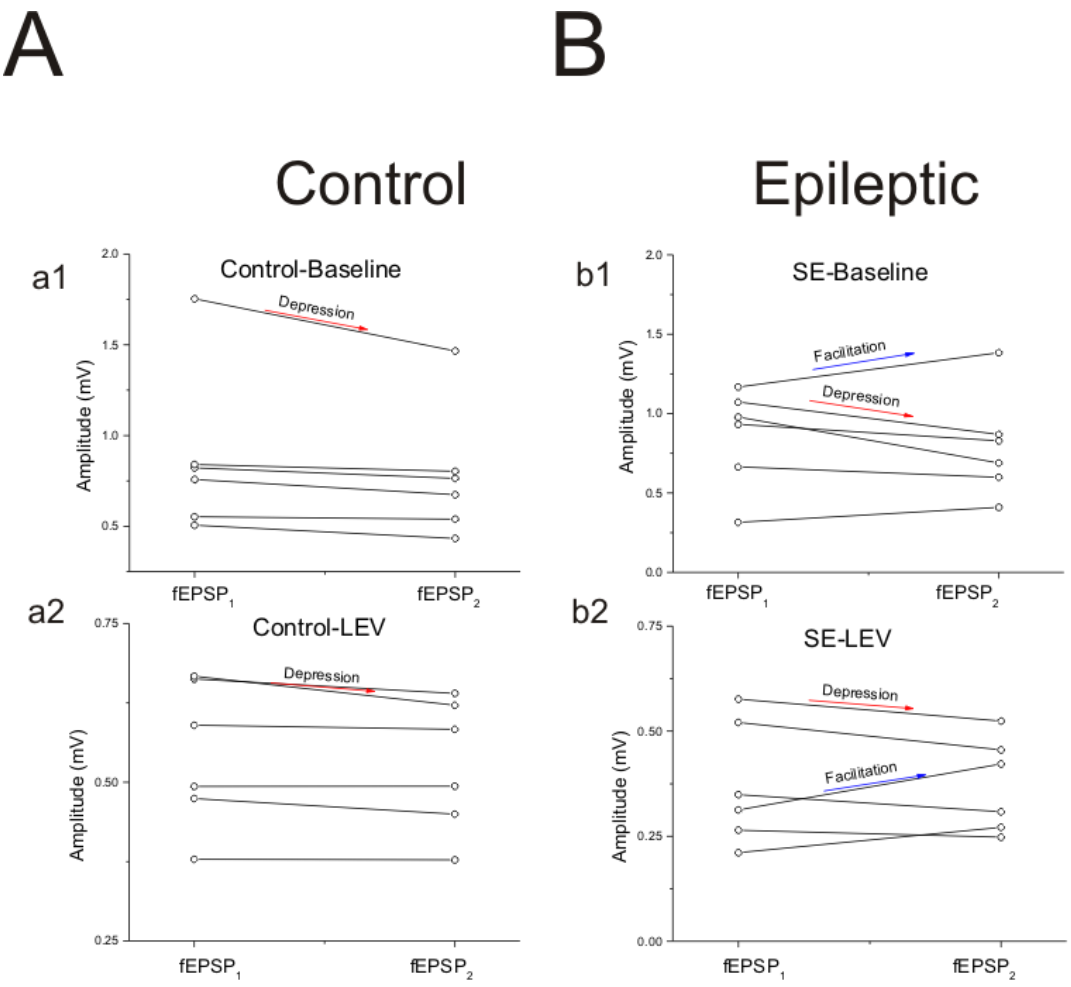

Figure 4. *Graphs representing changes in amplitude of first (fEPSP1) and second (fEPSP2) responses in the train of stimuli in both control (A) and epileptic groups (B) after treatment of slices with 300 μM LEV. A. Notice depression of fEPSP2 relative to fEPSP1 in non-treated slices and less depression after LEV treatment (a2). In contrast, slices in epileptic group exhibited both facilitation and depression*

We then normalized both fEPSPs in non-treated and LEV treated slices to the first fEPSP to investigate if the decay of depression is affected by LEV treatmen in control versus epileptic groups (Fig. 5). Analysis of the decay of fEPSP amplitudes normalized to fEPSP1 showed no significant differences (Kolgomorov-Smirnov Stat, p>0.05) of LEV treatment compared to baseline in control (Fig. 5A a1) and epileptic groups (Fig. 5 b1, Table 3). In addition, analysis of the decay (time constant) of depression after normalization to the first fEPSP in treated and non-treated revealed that in control group LEV treatment induced faster decay while in the epileptic group LEV induced slower decay of consecutive responses, however, these changes were not statistically significant with the current data and experiments performed (Table 4). However, after normalization to the first fEPSP, we then analyzed the effect of LEV on the PPR on each consecutive fEPSP in the train for both control and epileptic group*. As represented in graphs of Fig. 5A a2 and 5B b2, there was a significant change after LEV treatment for only the second normalized paired-pulse ratio since LEV treatment significantly increased the ratio relative to non-treated slices in both groups (Table 5).* No significant changes were detected for all the other consecutive rations.

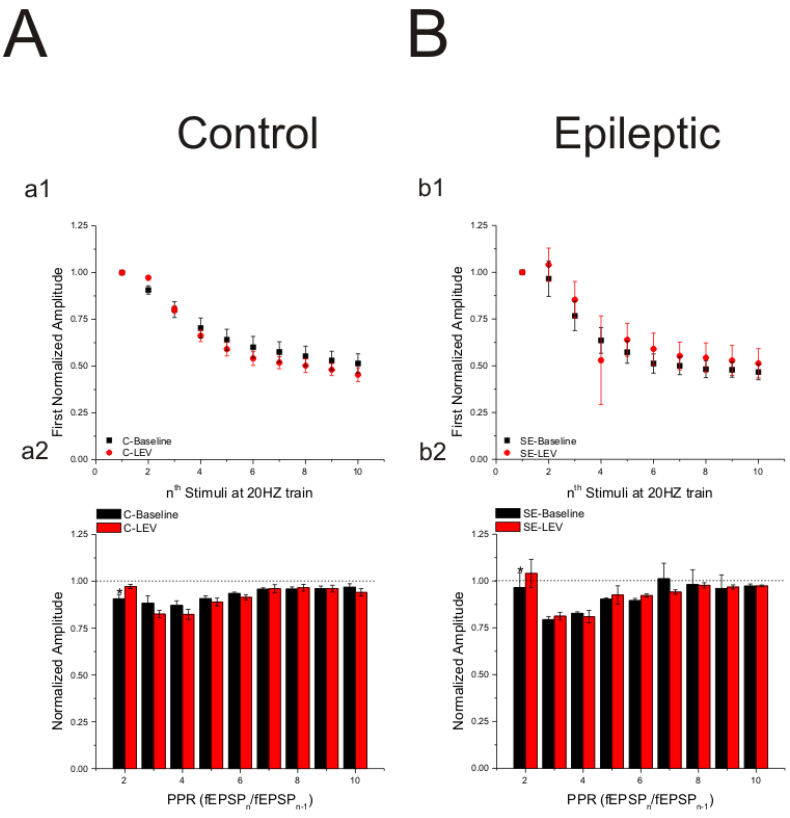

Figure 5. *Graph representing normalized data relative to first fEPSP amplitude in both control (A) and Epileptic (B) groups*. No significant changes were detected in the decay of amplitudes for subsequent fEPSP in the train of responses in groups after LEV treatment in both control (a1) and epileptic group (b1). Consecutive ratios were computed for fEPSP in the train revealing a significant increase (facilitation) for the first PPR1 in the train for both control (C-LEV, a2) and epileptic (SE-LEV, b2).

Table 3. Kolmogorov-Smirnov analysis for the amplitude of dentate gyrus repetitive stimulation on control and chronic epileptic rats (first normalization)

	N	Min	Q1	Median	Q3	Max	D	Z	Prob>|D|
C-Baseline	9	0.51433	0.54125	0.60108	0.75255	0.90692	0.33333	0.15713	0.73011
C-LEV	9	0.45405	0.49113	0.54122	0.73193	0.9723			

	N	Min	Q1	Median	Q3	Max	D	Z	Prob>|D|
SE-Baseline	9	0.65433	0.67554	0.71755	0.86937	1.0563	0.11111	0.05238	1
SE-LEV	9	0.65842	0.67034	0.69928	0.84517	1.05568			

Table 4 Paired samples *t*-test analysis for paired for Decay constant time (τ) analysis for amplitude normalized (first) of control vs SE group.

Decay constant first normalized

CONTROL

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|
Baseline		5	139.2175	45.43435	20.31886	0.54835	4	0.61262
LEV		5	122.1192	42.10787	18.83121			
	Difference		17.09827					

EPILEPTIC

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|
Baseline		6	96.04301	15.37903	6.27846	-0.20467	5	0.8459
LEV		6	99.29265	39.3223	16.05326			
	Difference		-3.24964					

Table 5. Analysis of LEV effect on paired pulse ratio relative to normalized first fEPSP by Student Paired *t*-test for second fEPSP ($fEPSP_2/fEPSP_1$ normalized) in control and epileptic group.

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|
C-Baseline	peak2	6	0.90692	0.05572	0.02275	-4.10992	5	0.00926
C-LEV	peak2	6	0.9723	0.02853	0.01165			
	Difference		-0.06538					

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|
SE-Baseline	peak2	7	0.96539	0.21025	0.07947	-2.9756	6	0.02478
SE-LEV	peak2	7	1.04032	0.19871	0.07511			
	Difference		-0.07493					

We then normalized fEPSP amplitudes in the train ($fEPSP_{3-10}$) relative to the second $fEPSP_2$ to analyze whether rate of decay of fEPSP amplitudes differs after treatment with LEV in both control and epileptic groups (Table. 6). No significance changes were detected by Kolgomorov Smirnov statistical analysis of distributions of normalized fEPSP relative to second fEPSP. In addition, we compared changes in decay of fEPSP amplitudes in the train without treatment (baseline in control versus epileptic group) (Fig. 6A) and in slices treated with LEV in both groups (Fig. 6B). Statistical analysis revealed that although fEPSP amplitudes decay faster in epileptic groups (both untreated and LEV treated groups) (Table 7), changes in time constant (τ) of rate of decay were not significant by student *t*-test.

Table 6. Kolmogorov-Smirnov analysis for the normalized amplitude fEPSP in train relative to second fEPSP after LEV inn control and chronic epileptic groups.

	N	Min	Q1	Median	Q3	Max	D	Z	Prob>|D|
SE-Baseline	9	0.48798	0.50167	0.53272	0.72575	1	0.55556	0.26189	0.12587
SE-LEV	9	0.58304	0.61355	0.64425	0.78486	1			

	N	Min	Q1	Median	Q3	Max	D	Z	Prob>|D|
C-Baseline	9	0.56681	0.59704	0.6629	0.82919	1	0.55556	0.26189	0.12587
C-LEV	9	0.46789	0.50597	0.55686	0.753	1			

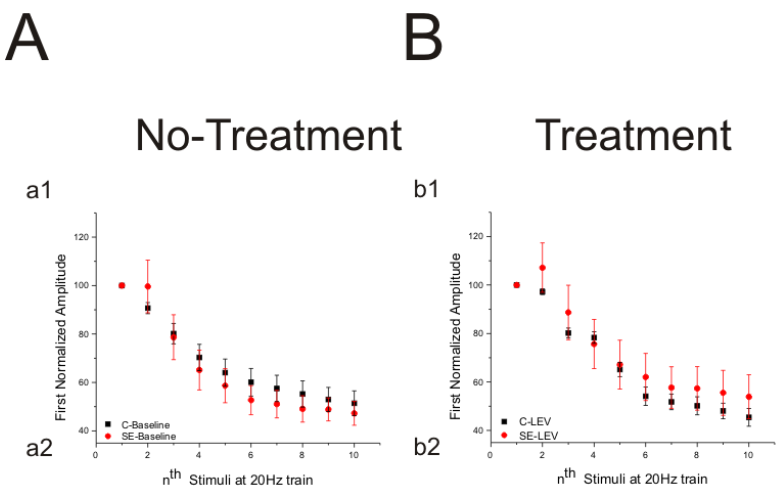

Figure 6. LEV did not affect the rate of decay of fEPSP following the second response when compared no treated slices in control versus epileptic group (A) and LEV-treated slices in control versus epileptic group (B). fEPSPs in the train were normalized to first response (fEPSP1) .

Table 7. Decay constant time (τ) analysis for amplitude normalized (first) of control vs SE group.

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|
Baseline	control	5	139.2175	45.43436	20.31886	2.5612	4	0.06256
Baseline	SE	5	92.58236	14.34652	6.41596			
	Difference		46.63509					

		N	Mean	SD	SEM	t Statistic	DF	Prob>|t|
LEV	Control	6	124.3813	38.06786	15.54114	1.09262	5	0.32439
LEV	SE	6	99.29265	39.3223	16.05326			
	Difference		25.08867					

4. Quantification of the plasma level of LEV for HPLC assays

In order to assess plasmatic levels of LEV after systemic intraperitoneal administration of LEV we developed High Pressure Liquid Chromatography (HPLC) assays to measure the plasma concentration of LEV in separate group of animals. LEV HPLC assays were performed using column BetaBasic-18 of particle size 5 μm (Thermo Electron Corporation) and eluent 5% Acetonitrile in water with flow 0.25 ml/min for 15 min and retention time of 8.8 min. LEV was monitored at 205 nm. For analysis of HPLC chromatogram was used Empower software (Waters). HPLC analysis was conducted using Waters 2695 separation module equipped by 2996 PhotoDiode detector. For these experiments, LEV (dissolved in saline solution) was injected intraperitoneally at doses of 100mg/kg (n=6) or 200mg/kg (n=6). Animals were sacrifice after 1, 3 or 7 days after treatment blood was collected and plasma was separated using standard procedures. Concentration of plasmatic LEV in samples were then analyzed HPLC analysis using LEV analytical grade from Sigma-Aldrich as standard for HPLC. Plasma isolated from animals injected with physiological saline (n=2) was used as control. Our results indicate that in animals injected with 100 mg/kg LEV reached highest plasma levels (0.69 μg/dl) at 5 days following injection and then declined to 0.53 μg/dl 7 days after LEV injection (Figure 7, Table 8) while at a dose of 200 mg/kg of intraperitoneal LEV, plasmatic levels reached a maximum at 5 days (0.67 μg/dl), but levels continue high after 7 days of injection (0.65 μg/dl) (Figure 7, Table 8). It is important to notice that levels at 5 days were similar with both doses of 100 mg/kg and 200 mg/kg, but higher dose 200 mg/kg induced a long-lasting increase in plasma LEV concentrations.

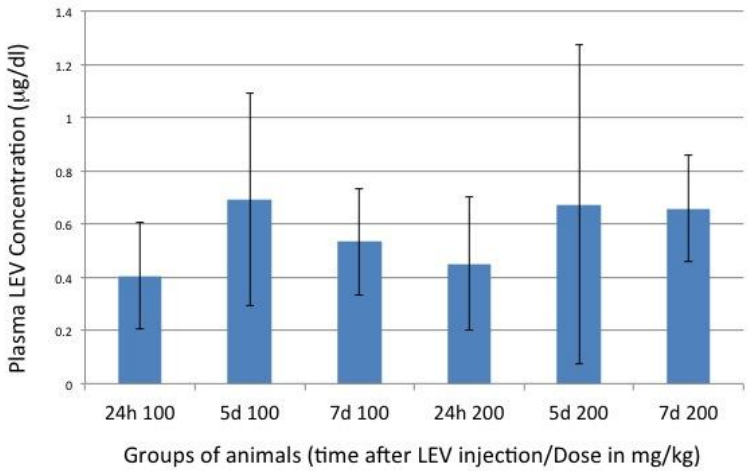

Figure 7. Graph representing plasmatic concentration of LEV after intraperitoneal injection of 100 mg/kg and 200 mg/kg. Blood samples were collected at 24hours, 5 days and 7 days after LEV administration and plasmatic LEV concentration was measured using HPLC.

Table 8. Plasmatic LEV concentration measured by HPLC.

LEV Plasmatic Concentration (µg/dl)	Experimental groups	*error*
0.404689	24h 100 mg/kg (n=3)	0.2
0.693338	5d 100 mg/kg (n=3)	0.4
0.532885	7d 100 mg/kg (n=3)	0.2
0.451048	24h 200 mg/kg (n=3)	0.25
0.673199	5d 200 mg/kg (n=3)	0.6
0.656432	7d 200 mg/kg (n=3)	0.2

5. Analysis of the expression of synaptic vesicle proteins during epileptogenesis.

The involvement of SV2A on epilepsy is indicated by 3 facts: (1) SV2A is the sole molecular target for the antiepileptic drug levetiracetam LEV[9], (2) SV2A knockout mice exhibit severe seizures thought to be associated with increased probability of transmitter release[10,11], and (3) down-regulation of SV2A has been reported in epilepsy. The exact antiepileptic mechanism of LEV remains elusive. Hence, the empirical use of this drug has limited the rational development of bioassays to search for other compounds displaying similar or more potent actions. Moreover, given the lack of functional assays to probe whether LEV modifies SV2A function, the exact binding site of LEV on the structure of SV2A has not been discovered yet. As described below, SV2A levels changes in both experimental models and human suffering epilepsy.[12-14] However, it is not clear that SV2B or SV2B levels changes in epilepsy. This is important because patch-clamp experiments during the first year revealed that LEV is still effective to reduced excitatory postsynaptic currents to granule cells in SV2A knockout mice, opening a question of whether LEV uses alternative synaptic vesicles to enter neurons or exert pharmacological effects. In order to analyze SV2A, SV2B and SV2C expression changes during the epileptic process we performed immunohistochemistry, western blotting and real-time quantitative PCR using TaqMan assays and the delta-delta CT relative quantification approach with Gapdh as the normalizing gene (Fig 8). Our data revealed a significant down regulation of SV2A immunostainings in mossy fibers in *stratum lucidum* (Fig. 8A) and dentate gyrus of chronically epileptic rats (Fig. 8B) that correlate with a 61% down-regulation of SV2A proteins by Western Blotting at 10 days following *status epilepticus* (Fig. 8C). At 1 month after status epilepticus, SV2A protein levels were still significantly reduced at51% of control group. Down-regulation was also expressed in animals sacrificed at more than 2 months period after status epilepticus. Reduction of SV2A transcripts in qPCR was not as dramatic when compared to changes in protein expression (Fig. 8D) but this may indicate a compensatory change in transcript production or an increase in degradation of SV2A.

We then analyzed the expression of other proteins of the same family Sv2B and SV2C exhibiting high homology to SV2A. [15-18] Immunohistochemical analysis revealed that SV2B was not expressed in mossy fibers in neither control nor epileptic slices. SV2B expression was more apparent in stratum radiatum and other areas. We observed a marked down-regulation of SV2B in epileptic rats Western blotting assays (60% reduction, Fig. 9C) that was consistent with data of real-time qPCR assays (Fig. 9D). SV2B down-regulation persisted during the entire epileptic phases of this model. Immunofluorescence assays for SV2C revealed a pattern of distribution similar to SV2A expression in that expression is high in mossy fibers and dentate gyrus in slices from control and chronically epileptic animals (Fig. 10A, B). Analysis of SV2C by Western blotting showed that despite initial reduction 24h following *status epilepticus*, SV2C, a protein that exhibit a distribution similar to SV2A exhibited an intriguing up-regulation at 10 days following status epilepticus. These findings on SV2C protein expression correlate with upregulation in the SV2C transcripts as shown by qPCR (Fig. 10D). These data indicate that down-regulation of SV2A expression contrast with up-regulation of SV2C in the pilocarpine model of epilepsy. Although, binding of LEV to SV2C has not been demonstrated, homology of these two proteins is very high and it is possible that during the epileptogenic process additional modifications of the SV2C proteins (*e.g.* alternative splicing) may allow these SV2C proteins to add as “alternative” targets for LEV and may preserve the antiepileptic effect of LEV either acting as LEV transporters or molecular pharmacological targets affecting presynaptic release of glutamate at excitatory synapses.

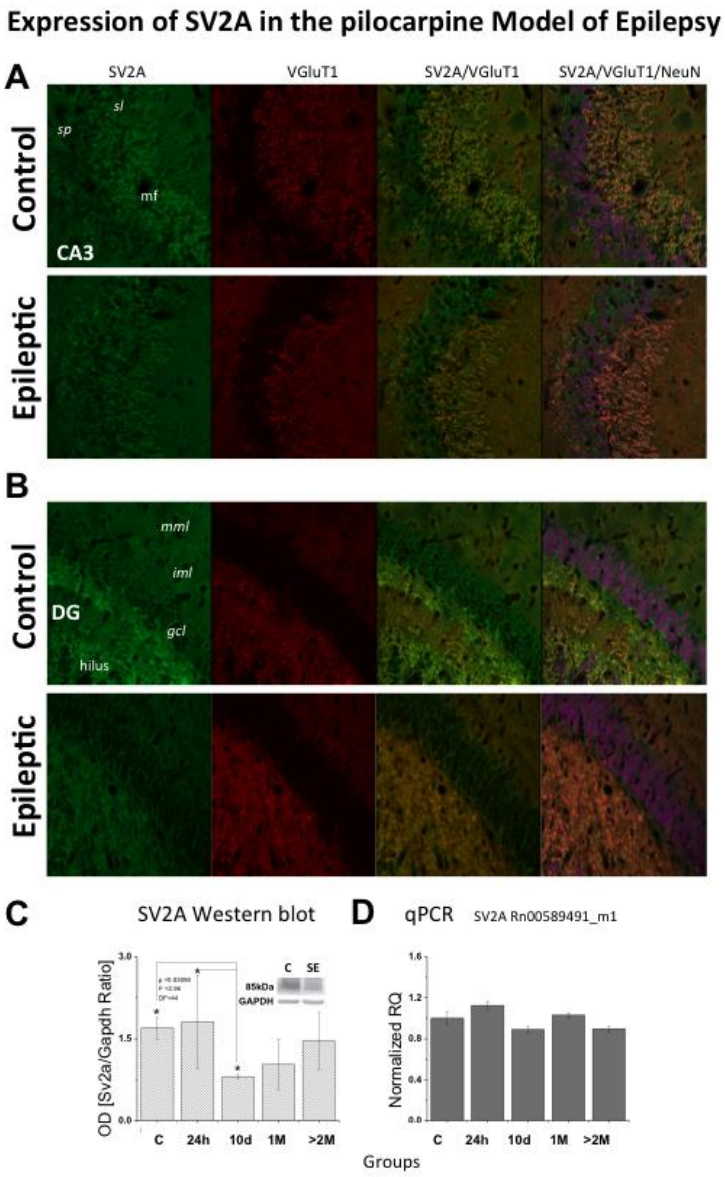

Figure 8. *SV2A is down-regulated in chronically epileptic rats*. A. Immunofluorescence assays indicating expression of SV2A in mossy fiber and dentate gyrus in control animal and a marked reduction of staining in epileptic rat while no detectable changes were found for expression of vesicular glutamate transporter type 1 (VGluT1). C. Western blotting showed a significant reduction of SV2A expression at 10 days and subsequent down-regulation at 1month and more than 2 months after *status epilepticus*. D. Data from real-time PCR (TaqMan assays) correlate with SV2A protein down-regulation in chronically epileptic rats. Normalizing gene: Gapdh.

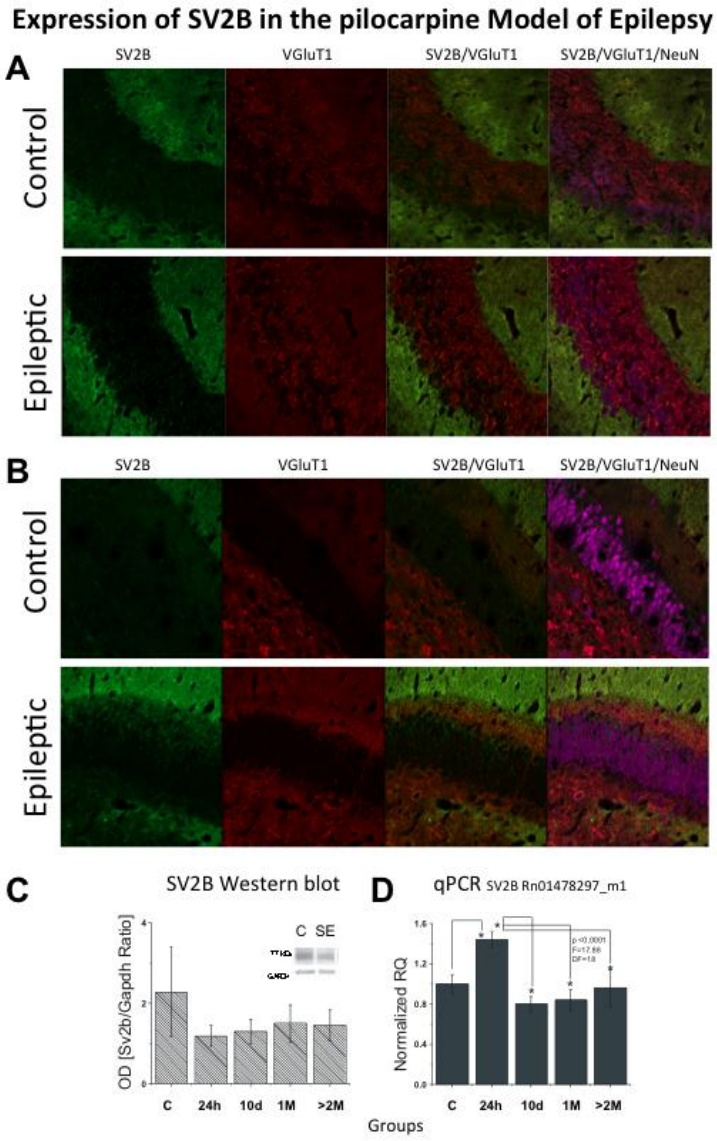

Figure 8. *SV2B is down-regulated in chronically epileptic rats*. A. Immunofluorescence assays indicating that SV2B is not expressed in mossy fibers in neither control animal nor epileptic rat while vesicular glutamate transporter type 1 (VGluT1) was epressed in mossy fibers (red staining). Counterstaining of granule cells in dentate gyrus was obtained using C. Western blotting showed a significant reduction of SV2A expression at 10 days and subsequent down-regulation at 1month and more than 2 months after *status epilepticus*. D. Data from real-time PCR (TaqMan assays) correlate with SV2A protein down-regulation in chronically epileptic rats. Normalizing gene: Gapdh.

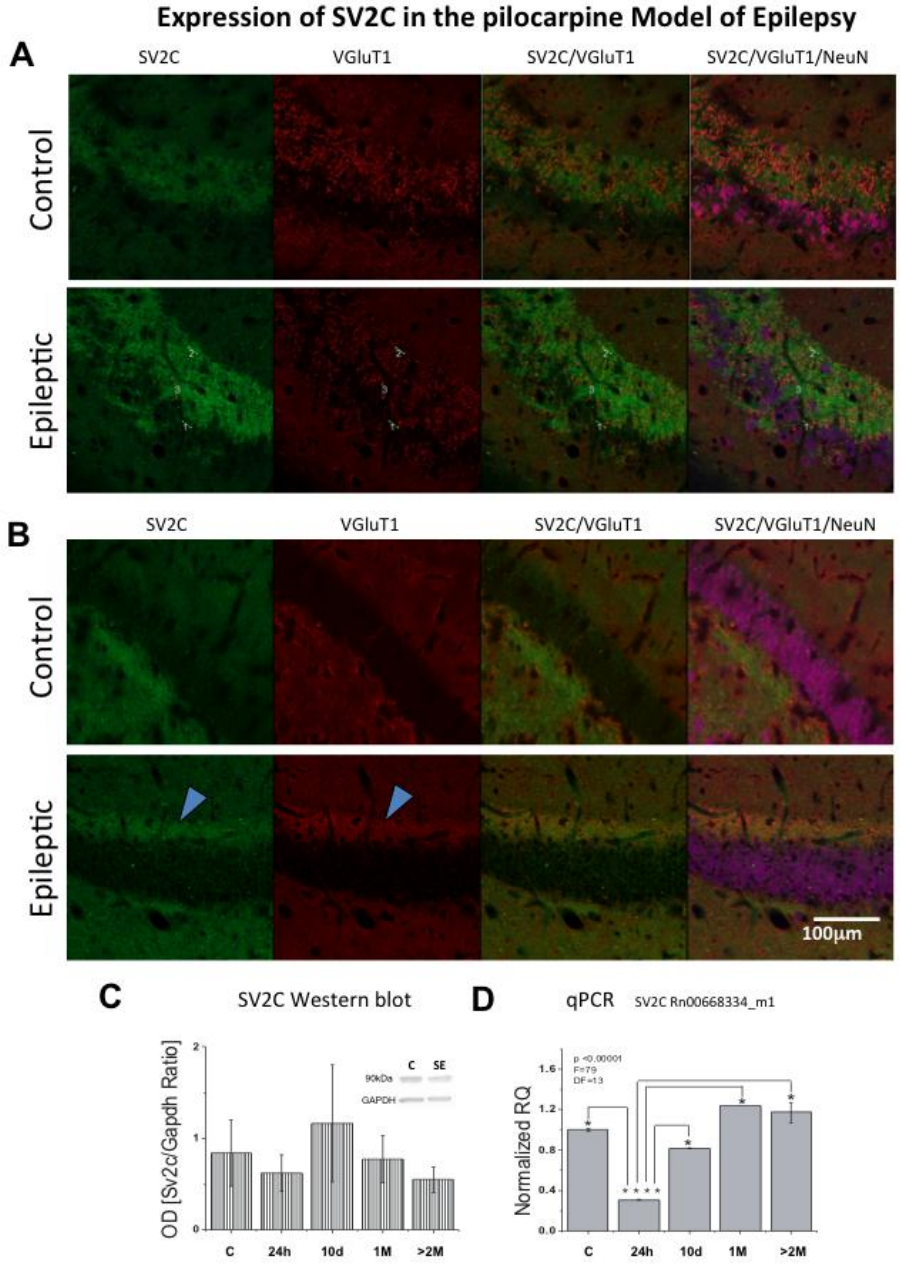

Figure 8. *SV2C expression was up-regulated in chronically epileptic rats*. A. Immunofluorescence assays indicating expression of SV2C is high in mossy fiber and dentate gyrus in both control and epileptic rat while no detectable changes were found for expression of vesicular glutamate transporter type 1 (VGluT1). C. Western blotting showed a significant up-regulation of SV2C expression at 10 days and subsequent down-regulation at 1month and more than 2 months after *status epilepticus*. D. Data from real-time PCR (TaqMan assays) indicate a down-regulation at 24h, but subsequent increases that were significant at 1 montha nd more than 2 months after *status epilepticus* (chronic epileptic phase). Normalizing gene: Gapdh.

Milestones: The following milestones were accomplished during year 1.

(a) LEV was effective in inhibiting the excitability of slices as measured by population spike amplitude in both control and epileptic rats
(b) LEV significantly reduced excitatory synaptic transmission onto granule cells in dentate gyrus.
(c) We discover that although SV2A is down-regulated SV2C is upregulated 10 days after *status epilepticus.* We discovered that SV2C expression in hippocampus followed similar distribution as SV2A. SV2C may represent novel targets for LEV in the epileptic tissue.

KEY RESEARCH ACCOMPLISHMENTS:

1- LEV was effective in inhibiting the excitability of slices as measured by population spike amplitude in both control and epileptic rats
2- LEV significantly reduced excitatory synaptic transmission onto granule cells in dentate gyrus.
3- We discover that whole SV2A are SV2B are down-regulated,SV2C expression is upregulated after *status epilepticus.* We discovered that SV2C expression in hippocampus followed similar distribution as SV2A and may represent addition al targets for LEV action in epilepsy.
4- LEV plasmatic concentration was measured using a newly developed assay for LEV detection using HPLC. Injection of 100 and 200 mg/kg of LEV resulted in plasmatic concentration of approximately 0.6 μg/dl 5 days after injection.

REPORTABLE OUTCOMES:

National Meetings: Data from electrophysiological and pharmacological experiments were presented in a scientific meeting in 2012 (1) and new data will be presented in another meeting in 2013 (2).

1) *Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and pilocarpine-induced epilepsy.* **E. G. Sanabria,** L. F. Pacheco, L. M. Rambo, J. M. Rodriguez, C. Upreti, **P. K. Stanton**. Society for Neuroscience Meeting that will be held October 13 - 17, 2012, in New Orleans, LA (Abstract in Appendix 1).

2) *Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats.* **E. G. Sanabria**, L. Pacheco, J. Zavaleta, F. Shriver, L. M. Rambo, C. Upreti, **P. K. Stanton**. Society for Neuroscience Meeting that will be held in San Diego, CA, Nov 9-13, 2013 (Abstract in Appendix 2)

CONCLUSIONS:

We have obtained key data indicating that chronic slice treatment with LEV can reduce excitability and reduce excitatory synaptic transmission in hippocampal slices obtained from control and epileptic rats. These are significant findings because during the course of epilepsy LEV pharmacological target SV2A is down-regulate. Therefore, current data open two possibilities: (a) remaining SV2A receptors are still functional for LEV action and their activation is still effective in control presynaptic release of glutamate, overall excitability and ultimately seizures in epilepsy and/or (b) the action of LEV is independent of SV2A binding, but SV2A may act as a carrier/transport that mediates LEV molecules to enter the interior of the presynaptic boutons and act on secondary targets. Hence, even down-regulate, SV2A can bind and internalize enough LEV to act effectively in another intracellular targets or pathways. Although LEV has been shown to act on other targets like presynaptic Ca^{2+} channels[19,20], binding experiments only confirm SV2A as a sole molecular receptor. Additional studies are necessary to pin-point and differentiate the role of SV2A in mediating the antiepileptic effect of LEV as receptor-effector versus receptor-transporter molecular targets. Our data indicate that presynaptically acting drugs such as levetiracetam reduces hyperexcitability and inhibit presynaptic transmission in mesial temporal lobe epilepsy.

Significance: LEV is a novel antiepileptic drug that binds to presyanptic targets. However, the presynaptic mechanisms are still unclear and innovative research paradigms need to be deployed to understand how this drug work in order to design similarly effective or better versions for the treatment of pharmaco-resistance epilepsy. We found that expression of LEV targts are down-regulated in epilepsy but expression of homologous protein SV2C is upregulated 10 days after status epilepticus and transcripts are upregulated during the chronic phase of the model. Sv2C my represent novel targets for LEV and similar drugs acting on presynaptic terminals to control release of glutamate. In this project will elucidate the presynaptic mechanisms of action of LEV and other antiepileptic drugs as a strategy to accelerate the development of novel antiepileptic drugs acting in presynapses. In Year 3, we will assess if treatment with LEV can restore molecular deficiencies caused by epilepsy in this animal model of mesial temporal lobe epilepsy (including down-regulation of SV2A and mGluR2. Understanding abnormalities of presynaptic terminals during epileptogenesis is critical to elucidate the pathogenesis of pharmacoresistant epilepsy.

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APPENDICES

Appendix #1

Abstract submitted to the Society for Neuroscience Meeting that will be held in Nov 9-13,

San Diego, California

Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats. **E. G. SANABRIA**[1], L. PACHECO[1], J. ZAVALETA[1], F. SHRIVER[1], L. M. RAMBO[2], C. UPRETI[3], **P. K. STANTON**[3];

Control/Tracking Number: 2013-S-12910-SfN

Activity: Scientific Abstract
Current Date/Time: May 9, 2013 12:07:38 PM EDT

Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats.

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Abstract:
The presynaptic target for Levetiracetam (LEV) has been identified as synaptic vesicle SV2A proteins in presynaptic terminals; however, the mechanisms of LEV's antiepileptic action remain unclear. Previous studies have shown a reduction of SV2A expression in both animal models and human suffering mesial temporal lobe epilepsy (MTLE). However, in vivo treatment with LEV appears to be still effective in those conditions in ameliorating seizures. In this study, we evaluated the in vitro effects of LEV on excitability and excitatory synaptic transmission in the pilocarpine model of mesial temporal lobe epilepsy (MTLE). In this study, we investigated the action of LEV on (a) population spikes recorded in CA1 area and (b) excitatory synaptic transmission onto dentate gyrus of control versus chronically epileptic rats obtained by the pilocarpine model of MTLE. For this purpose, we used extracellular potential recordings in acutely dissociated slices. Slices were pre-incubated in 300 microM of LEV for 3 hours prior recordings. LEV was also applied in the bath during recording sections. Field excitatory postsynaptic potentials (fEPSP) were evoked by different paradigms of repetitive stimuli of perforant path (e.g. 10@20Hz). Pre-incubation with LEV induced a 20% and 10% reduction in amplitude of CA1 population spikes in slices from control and epileptic rats respectively relative to non-treated slices. LEV induced a 37.2% and 49% significant reduction in the amplitude of the summated fEPSPs in a 20Hz train evoked by perforant path stimulations in both control and epileptic groups respectively ($df=9$, $p< 0.0001$ by paired T-test) compare to baseline. Significant changes were also detected in the first four fEPSP responses in the train with a non-significant reduction of remaining 6 fEPSPs (ANOVA repetitive Test, $p<0.01$ for both groups followed by pairwise Tukey post-hoc test). These results indicate that LEV is effective in reducing in vitro excitability and excitatory synaptic transmission in both control and epileptic groups (despite possible changes in SV2A expression). Further studies are in progress to determine presynaptic mechanisms involved in this inhibitory effect.

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Appendix #2

Poster presented at the Society for Neuroscience Meeting New Orleans, Oct 16, 2012

Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and mesial temporal lobe epilepsy. ***E. G. SANABRIA**[1], L. F. PACHECO[2], L. M. RAMBO[2], J. M. RODRIGUEZ[2], C. UPRETI[3], **P. K. STANTON**[4].

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Presentation Title: Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and mesial temporal lobe epilepsy.

Presentation time: Tuesday, Oct 16, 2012, 2:00 PM - 3:00 PM

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Abstract: Levetiracetam (LEV) is a new type of antiepileptic drug (AED) exhibiting selective seizure protection in chronic animal models of epilepsy. LEV binds selectively to the synaptic vesicle protein SV2A, indicating a presynaptic site of action to counter hyperexcitability. In this study, we evaluated the in vitro effects of LEV on excitatory synaptic transmission in the pilocarpine model of mesial temporal lobe epilepsy (MTLE). It has been reported that expression levels of SV2A decline during the course of human epilepsy and in experimental MTLE. We hypothesized that LEV action may be differentially affected during epileptogenesis and in transgenic mice with altered SV2A expression. For this purpose, we assessed LEV effects on excitatory synaptic transmission in slices from pilocarpine-treated epileptic and control mice with different SV2A genotypes, by recording AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in dentate granule cells using whole cell patch-clamp recording. Different concentrations of LEV (5, 50 and 100 microM) were bath applied to evaluate effects on mEPSC frequency and amplitude. Double SV2A/SV2B knockout (KO) mice were not included in this study due to early life mortality. 14% of SV2A heterozygous KO mice exhibited spontaneous seizures (epileptic). LEV induced a significant decrease of mEPSC frequency in granule cells from SV2A wild-type (26% reduction) and heterozygous mice (37% reduction) when compared to pre-drug baseline. LEV (100 µM) failed to modify mEPSC frequency in ~ 60% of slices from SV2A KO mice, while a paradoxical increase of mEPSC frequency was detected in the rest of the slices. LEV still induced a significant decrease of mEPSC frequency (51.7% reduction, paired t-test, $P<0.05$) in slices from SV2A/SV2B (wild-type) mice sacrificed 2-4 months after status epilepticus. LEV exerted no significant effects on mEPSC amplitude in any group. Our findings indicate that LEV acts presynaptically to inhibit glutamatergic drive onto dentate granule cells in control and chronically epileptic mice, but that this effect is more pronounced in epileptic slices. Lack of SV2A expression occluded the inhibitory effect of LEV on excitatory transmission in a subset of animals, while a paradoxical increase of glutamate release was detected in the rest. Although LEV selectively binds SV2A in normal brain, it is possible that compensatory changes (i.e. abnormal splicing) of remaining SV2B and SV2C proteins may provide additional non-SV2A LEV binding sites in SV2A KO and in epileptic mice with significant implications for the development of novel LEV-like AEDs.

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